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Chitosan nanoparticles induced germination and auxin-related gene regulations in cotton (*Gossypium hirsutum* L.) using random forest-based multi-output regression analysis

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ABSTRACT

Chitosan nanoparticles (CNPs) are emerging nanobiostimulant known to enhance germination, stress tolerance, and hormonal regulation in plants, yet their role in auxin-related gene regulation in cotton remains unexplored. In this study, cotton seeds were exposed to varying concentrations of CNP (0–2.5 mg/L) under in vitro conditions. Seed germination was counted daily for a continuous 21-day period, followed by analysis using the GerminaR statistical package to generate various germination metrics. The germination metrics were analyzed by Random Forest-based multi-output regression analysis. Auxin-related gene expression profiling of ARF, AUX, and UGT was performed at two different concentrations and two time points, using qPCR normalized to His3. Taguchi design (TD) was employed for analyzing gene expressions to quantify factor significance and noise robustness through the S/N ratio. Results revealed maximum germination percentage (GRP) at 1.0 mg/L CNP. Concentrations above 1.5 mg/L CNP negatively impacted germination kinetics with increased germination speed (GSP) and coefficient of variation in germination (CVG). The multi-output regression analysis revealed the highest predictive performance for variance in germination time (VGT) with an R^2 score of 0.785, followed by 0.746 for the CVG. Gene expression profiling revealed significant upregulation of ARF and AUX genes with increasing concentration and time. Whereas UGT regulation was dependent on both time and concentration, with variable impact. The TD analysis identified concentration as most significant for ARF regulation, while time was significant for AUX and UGT expressions, confirming the role of CNPs as a bio-stimulant.

1. Introduction

Chitosan is a natural, biodegradable, less toxic, and biocompatible polymer, primarily derived from the deacetylation of chitin (Riseh et al., 2022; Suwanchaikasem et al., 2024). Although chitosan is also present in the cell walls of certain fungal species, but less than chitin (Ghormade et al., 2017). The commercial production of chitosan is done through the deacetylation of chitin using specific chitin deacetylase enzymes at high temperature (Younes and Rinaudo, 2015). It is available in different forms, but chitosan nanoparticles are of great significance due to their physical properties (Nandini et al., 2025). In agriculture and biological sciences, chitosan has been widely documented to enhance plant growth, antioxidant activity, and stress tolerance (Yadav et al., 2024), and slow release of encapsulated agrochemicals (Obeidat and Lahlouh, 2025), and mitigating stress resilience (Jan et al., 2025; Sorokin et al., 2021).

Auxins are phytohormones known to play a key role in plant growth and development, and control a wide range of physiological processes such as cell division, cell elongation, and cell differentiation (Gomes and Scortecci, 2021). Auxins are generally formed through the cellular tryptophan pathway or other parallel pathways, causing rapid changes in transcription of many genes (Gao et al., 2024). Indole-3-acetic acid

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(IAA) is the most prominent auxin hormone found in plants and is downregulated by multiple genes. Auxin Response Factors (ARF) mediating the transcriptional signal transduction, auxin influx carriers (AUX) facilitating cellular entry, and uridine diphosphate glucosyltransferases (UGT) regulating hormonal homeostasis through conjugation were selected to function as a diagnostic triad representing the critical nodes of the auxin regulatory network (Roosjen et al., 2018; Mateo-Bonmatí et al., 2021; Cancé et al., 2022; Chen et al., 2025). While the relationship between CNPs and IAA contents has been documented in different plants, like wheat (Li et al., 2019), tomato (Suarez-Fernandez et al., 2020), and Arabidopsis (Lopez-Moya et al., 2017), systematic studies elucidating the specific auxin-related gene regulatory networks in cotton are currently lacking. Gene expression analysis is crucial to understanding the mechanisms by which chitosan affects plant growth (Kumaraswamy et al., 2019) especially plants with high economic value.

Cotton is the leading industrial crop due to its uses in multiple industries, ranging from textiles to food. The quality parameters of cotton fiber are highly critical, and CNP has been reported for enhanced cotton fiber characteristics, physiological parameters, germination, and stress resilience (Wang et al., 2021) by reducing crystallinity and enhancing surface functionality through hydrogen bonding (Wang et al., 2021). However, factors such as application time, concentration, and the method of CNP application are also critical. Application of CNPs is generally employed by treating seeds as nano-priming (Nile et al., 2022) or treating plant parts as a foliar spray (Bhati et al., 2025). However, prolonged exposure to CNPs is difficult and can be done through hydroponic or plant tissue culture by incorporating them into the medium (Bulut et al., 2025). Application of CNPs for investigating the impact on seed germination and auxin-induced genes is essential for achieving optimal plant establishment and maximizing productivity (Sen and Das, 2024; Vardhan et al. n.d.). Keeping in view, cotton seeds were exposed to CNP for a prolonged time by incorporating different CNP concentrations in the culture medium. Therefore, the germination pattern and auxin regulation of cotton seeds were investigated by culturing on medium enriched with CNPs at different concentrations and exposure times. The germination data was recorded daily to understand the germination pattern using multiple germination metrics (Aasim et al., 2023).

Data analysis in the modern era of computation and data science is conducted with more sophisticated statistical tools, artificial intelligence-based machine learning (ML) models, or a hybrid approach combining multiple techniques. The germination metrics data generated in this study in response to multiple CNPs concentrations were analyzed with random forest-enabled multi-output regression analysis to predict multiple output (continuous) variables simultaneously (Wang et al., 2024). Multi-output regression aims to predict multiple real-valued output/target variables simultaneously. In multioutput regression, the outputs are dependent upon the input and upon each other, showing that the outputs are not independent of each other (Borchani et al., 2015; Beigaitė et al., 2022). It yields better predictive performance compared to the single-output methods (Han et al., 2012) by considering underlying relationships between the features and the corresponding targets, relationships between the targets (Kocev et al., 2009; Tuia et al., 2011), and may produce simpler models with a better computational efficiency (Kocev et al., 2009). Multi-step time series forecasting is a type of multiple-output regression where a sequence of future values is predicted, and each predicted value is dependent upon the prior values in the sequence. Furthermore, data related to gene expression profiling of auxin-related genes were computed with the Taguchi design (TD) for robustness of the model. The study was designed and investigated to understand complex biological processes, like germination and gene expression, using modern statistical tools and ML models.

2. Material and methods

2.1. Application of chitosan NPs and germination metrics

The chitosan NPs used in this study were procured from Sigma Aldrich, and a stock solution was prepared according to protocol (Bulut et al., 2025). The CNPs were used at different concentrations of 0, 0.25, 0.50, 1.00, 1.50, 2.00, and 2.50 mg/L by adding them directly to the culture medium before autoclaving the culture medium. The culture medium was prepared by adding 4.4 g/L Murashige and Skoog (MS) medium basal salts, including vitamins, 30.0 g/L sucrose, and the pH (approximately 5.8) was adjusted after adding CNPs. The culture medium was gelled with 6.5 g/L agar. All chemicals used for making culture medium were procured from Ducehfa Biochem. The seeds of cotton *Cv.* STN 468 was used as plant material for this study, and surface sterilized in our lab (Sivas University of Science and Technology) with a protocol established and reported (Özkat et al., 2025). The seeds were exposed to 0.1% $HgCl_2$ for 15 min, followed by three rinses, each for 5 min. Thereafter, seeds were placed in the jars filled with MS culture medium and placed in the growth room under controlled conditions of 23 °C temperature, 60.0% relative humidity. The growth room was equipped with white light-emitting diodes (LEDs) with light illuminance of 2000 Lux and 16 h light photoperiod. The data regarding germination were tabulated daily for 24 days, but data up to 21 days were used for analysis due to no further change in germination after 21 days. Thereafter, data were subjected to the GerminaR statistical package of the R statistical program, and seven different germination metrics were used for understanding the impact of CNPs on the germination process and seedling establishment (Aasim et al., 2023).

The germination percentage (GRP) refers to the percentage of seeds that completed the germination process.

$$GRP = \frac{\sum_{i=1}^{k} n_i}{N} \times 100$$

where n_i is the number of seeds germinated in the i^{th} time; N represents the total number of seeds in each experimental unit; k refers to the last day of germination evaluation.

MGT denotes the number of seeds germinated with respect to the number of seeds germinated at the time of evaluation.

$$MGT = \frac{\sum_{i=1}^{k} n_i t_i}{\sum_{i=1}^{k} n_i}$$

t_i is the time from the beginning of the experiment to the i^{th} observation.

The germination speed coefficient

$$GSP = \frac{\sum_{i=1}^{k} G_i}{\sum_{i=1}^{k} G_i X_i} \times 100$$

G_i is the number of seeds germinated in the i^{th} time, and X_i represents the number of days from sowing.

The germination uncertainty (UNC) is used to measure how evenly germination is spread out over time. When UNC values are low, it usually means that more seeds are germinating at the same time. In other words, low uncertainty indicates that germination is more concentrated. This index captures how spread out or clustered the germination is.

$$UNC = -\sum_{i=1}^{k} f_i \log_2 f_i \text{ with } f_i = \frac{n_i}{\sum_{i=1}^{k} n_i}$$

The germination synchrony (SYN) was originally developed to estimate the degree of overlapping of flowering among individuals in a population, and scores ranged from 0 to 1.

$$SYN = \frac{\sum_{i=1}^{k} C_{n_i,2}}{C_{\sum n_i,2}} \; with \; C_{n_i,2} = \frac{n_i(n_i-1)}{2}$$

The index of germination variance

$$VGT = \frac{\sum_{i=1}^{k} n_i(t_i - \bar{t})^2}{\sum_{i=1}^{k} n_i - 1}$$

The germination coefficient of variation

$$CVG = \left(\frac{\sqrt{VGT}}{MGT}\right) \times 100$$

2.2. Expression profiling

For gene expression of IAA-induced related genes, leaf samples were taken from in vitro grown plantlets (14 and 21 days). These time points were selected to distinguish between two distinct physiological stages: the early seedling establishment phase immediately following the cessation of rapid germination kinetics and the stable vegetative growth phase, as germination metrics showed no further significant changes after this period.

Approximately 100 mg leaf samples were cut and grinded with liquid nitrogen for total RNA isolation. When leaf samples were powdered, the WizPrep Plant RNA Mini kit was used to get total RNA by following the protocol provided by the manufacturer. The isolated RNA was subjected to reverse transcription to produce cDNA through the Wiz Script cDNA synthesis kit, following the manufacturer's protocol. For relative expression analyses, the His3 gene, validated as a stable reference gene in cotton leaf tissues under abiotic stress conditions (Wang et al., 2013) was used as an internal control for normalization. Relative expression of ARF (XM_016883232.2), AUX (XM_016815584.2), and UGT (XM_016845129.2) genes were performed by normalizing to His3 expression and was calculated as a ratio to untreated cotton leaves at the same point. Primers used for the expression profile are given in Table 1.

For the qPCR reaction, 5 µL iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 0.5 µL forward (10 µM) and 0.5 µL (10 µM) reverse primer, 0.5 µL cDNA, and 3.5 µL PCR-grade water were used. Thermocycle conditions were maintained as initial denaturation at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 15 s, and annealing together with amplification at 50 °C for 1 min. Following amplification, the melt curve analysis at 65–95 °C with 0.5 °C increment was performed. Readings were taken using the AriaMx Real-time PCR System FAM and ROX channels. Raw data were analyzed according to the Livak and Schmittgen, 2001 method and were normalized to the His3 gene. Relative expressions of AUX, ARF, and UGT genes of treated samples were analyzed, compared to non-treated samples at two different time points (14th and 21st day) and two different chitosan concentrations of 1 mg/L (KZ1) and 2 mg/L (KZ2). These specific concentrations were selected based on the germination kinetics data, where 1.0 mg/L (KZ1) represented the optimal physiological threshold yielding maximum germination efficiency, while 2.0 mg/L (KZ2) was selected as a representative supra-optimal concentration to investigate the molecular basis of the observed inhibitory stress responses.

Table 1
Primers used for qPCR for relative expression.

Primers	Forward primer (5'-3')	Reverse primer (5'-3')
GsHis3	gaagcctcatcgataccgtc	ctaccactaccatcatggc
ARF	cctgagcaggttgcttttattcaat	aattaggggtaactcggcttc
AUX	aacttactctggcatggtgg	gaatccctgacagcatcccca
UGT	ttcatttgggttattagag	ttccacccacagtgtgtcaa

2.3. Statistical and Taguchi design analysis

Data generated for gemination metrics and time series was generated by the GerminaR statistical package. One-way analysis of variance (ANOVA) of both germination indices and gene expression was subjected to statistical analysis using the Minitab Statistical program. The difference between the means was compared with the Tukey test. Whereas the Taguchi design (TD) analysis of gene expression data was also performed with the Taguchi design (Kisacik and Aasim, 2025).

2.4. Multi-output regression analysis

In this study, a multi-output regression framework was used to predict germination metrics. Instead of training separate models for each trait, a single model was used to predict all targets at once, which helps capture correlations among traits for improved generalization (Wang et al., 2024). This strategy also reduces redundant computation compared to building individual models (Tsoumakas and Vlahavas, 2007; Borchani et al., 2015). Random Forest algorithm was selected due to its ability to handle nonlinear responses and complex interactions without strict distributional assumptions (Hoffman et al., 2021). Each tree in the ensemble was trained on bootstrap samples, and predictions for all output traits were averaged across trees. Hyperparameter tuning was done for the number of trees and the maximum depth, and the best model was selected by minimizing the sum of root mean square errors (RMSEs) for all traits (eq 8). This criterion ensures balanced accuracy so that improving one trait does not compromise others.

$$\theta^* = \; argmin_\theta \sum_{j=1}^{m} RMSE_j(\theta)$$

where θ denotes the hyperparameters of the Random Forest model.

2.5. Feature importance analysis

To optimize the most significant input factor, the ML result was further computed with feature importance analysis. For this purpose, chitosan concentrations and time were considered as input factors. The values were taken from the RF-based multi-output regression analysis, and the contribution of each predictor was computed as the normalized total reduction in RMSE for all trees in the ensemble (Breiman, 2001). The feature importance of the individual trait was performed, followed by calculating the mean and standard error. While all germination metrics were considered as a single factor due to their interdependencies on each other, a single feature importance graph was constructed by summarizing them. The daily germination data were labelled as D_0 to D_{20}, where D_0 refers to the first day after sowing, and D_{20} is the last day of data after three weeks.

3. Results

3.1. Time series analysis

The cumulative germination data recorded daily for all chitosan concentrations are presented in Fig. 1. Results revealed a clear concentration-dependent response over 20 days, and maximum germination was recorded at 1.0 mg/L CNP, followed by a sharp decline at higher doses. Maximum germination was recorded for the first ten days when 1.0% chitosan was applied. Whereas supplementation of 0.25 and 0.50 mg/L was quite slower than 1.0 mg/L, but still faster than the control. Supplementation of higher CNP concentration exerted a negative impact on the germination process, resulting in delayed or suppressed germination. Overall average germination percentage was

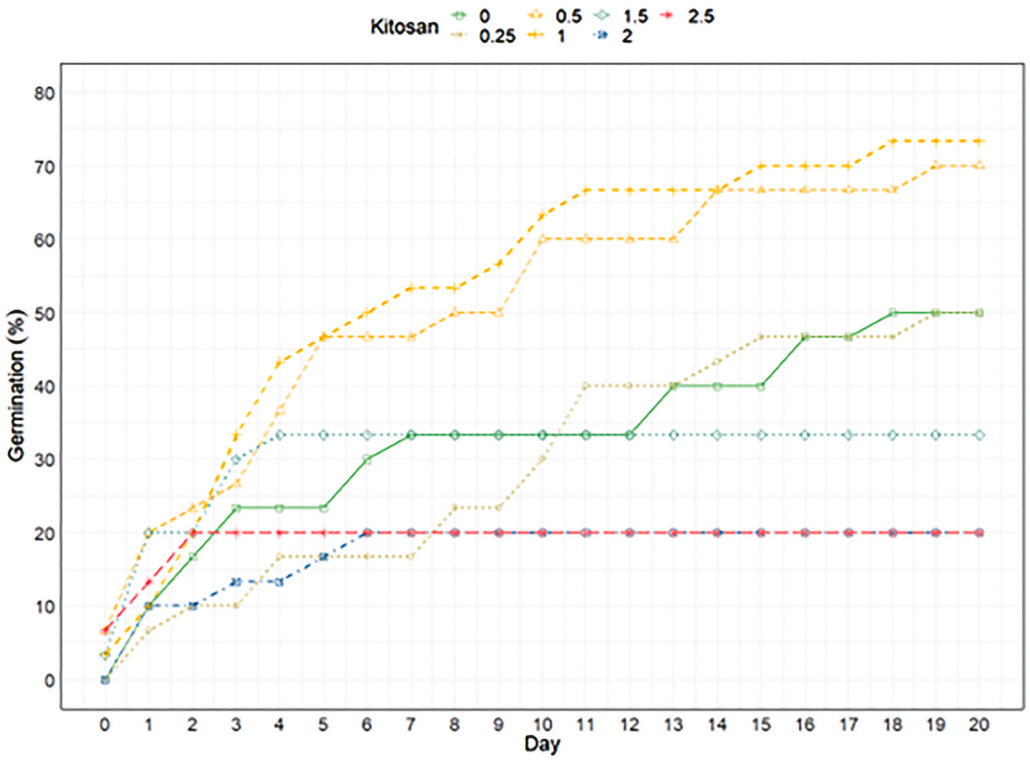

Fig. 1. Time series analysis of germination indices in response to CNP concentration.

around 60.0% at 1.0 mg/L, 30.0% for the control, 35–45% by lower concentrations, and 20.0% at doses above 1.5 mg/L. Seeds germinated after 10 days exhibited at 1.0 mg/L CNP, while enhanced germination in the first five days, followed by stabilization at lower concentrations (0.25 and 0.50 mg/L) was observed (Fig. 2).

3.2. Germination index analysis

Results of germination index (Table 1) revealed the significant impact of CNPs concentration, and GRP ranged from 20.0% to 73.33%. Supplementation of lower CNP (0.5 and 1.0 mg/L) concentrationsnotably improved GRP (70.0% and 73.33%) and resulted in stable and uniform germination. Whereas higher CNP concentration (1.5 mg/L or above) resulted in higher GSP and CVG, indicating delayed and less synchronized germination. A similar trend was recorded for both GSP and CVG, with a maximum scored were recorded at 2.5 mg/L and a minimum at 0.5 mg/L CNP. The MGT scores ranged from 1.0 to 7.93 and decreased with increased CNP concentration. Results further revealed that higher UNC and VGT scores were recorded from control and medium supplemented with 0.25–1.0 mg/L CNP. Further increase of CNP resulted in lower UNC and VGT scores. Whereas variable impact of CNP concentrations was recorded for SYN, with maximum from 2.0 mg/L (0.33) and 1.5 mg/L (0.17) CNP. germination metrics. These trends show that CNP concentration disrupts germination kinetics and follows a distinct optimum-decline pattern rather than a linear trend (Table 2).

The heatmap analysis presented in Fig. 2 exhibited the variable correlations between the germination metrics. The maximum correlation was recorded between GRP and UNC (0.96), and between GRP and MGT (0.69). On the contrary, a negative correlation between GRP and MGR, and between GRP and GSP was recorded as −0.76. Whereas an inverse relationship between MGT and MGR (-0.88). Interestingly, a complete and perfect correlation between MGR and GSP was also observed (1.00).

3.3. Multi-output regression analysis

The results of multi-output regression using the RF model are presented in Table 3. Results revealed variable R^2 scores for each germination trait and ranged from 0.281 to 0.785. Comparative analysis of all germination metrics exhibited the order of maximum to minimum as VGT (0.785), UNC (0.746), MGT (0.699), GRP (0.683), GSP (0.512), SYN (0.281), and CVG (0.229). Whereas results of RMSE and MAE scores were ranged from 0.185 to 31.267 and 0.072–21.991, respectively. The highest errors (both RMSE and MAE) were recorded for GSP and CVG. Whereas the minimum scores of both error metrics were observed for UNC and SYN.

3.4. Feature importance analysis results

The results of feature importance presented in Fig. 4 exhibit CNP as the most dominant factor compared to the individual time-based germination variables. The associated mean error reflects the heterogeneity, indicating the impact of CNP as trait-dependent. Investigation of time-dependent germination metrics exhibited a coherent temporal structure in their importance. Germination metrics during the early stage (D_0 to D_2) exhibited high importance, indicating the strong impact on final germination outcomes along with speed-related metrics. Whereas D_3 to D_{10} displayed relatively slow but exerted an impact on synchronization and cumulative germination progression. The germination between D_{10} and D_{20} was slow, with comparatively less impact on overall germination and other interdependent metrics.

To elucidate the molecular mechanisms underlying the observed germination responses, the relative expression of three key auxin-related genes (AUX, ARF, and UGT) was analyzed at two representative chitosan nanoparticle concentrations (KZ1 and KZ2) and at two times (14 and 21 days). The expression of the auxin influx carrier gene, AUX, showed a time and concentration-dependent response. AUX gene expression at KZ1 concentration was similar to that of the control at the 14th day, but increased significantly at the 21st day, with more than quadrupled expression. At 2.0 mg/L, the increment in expression was less

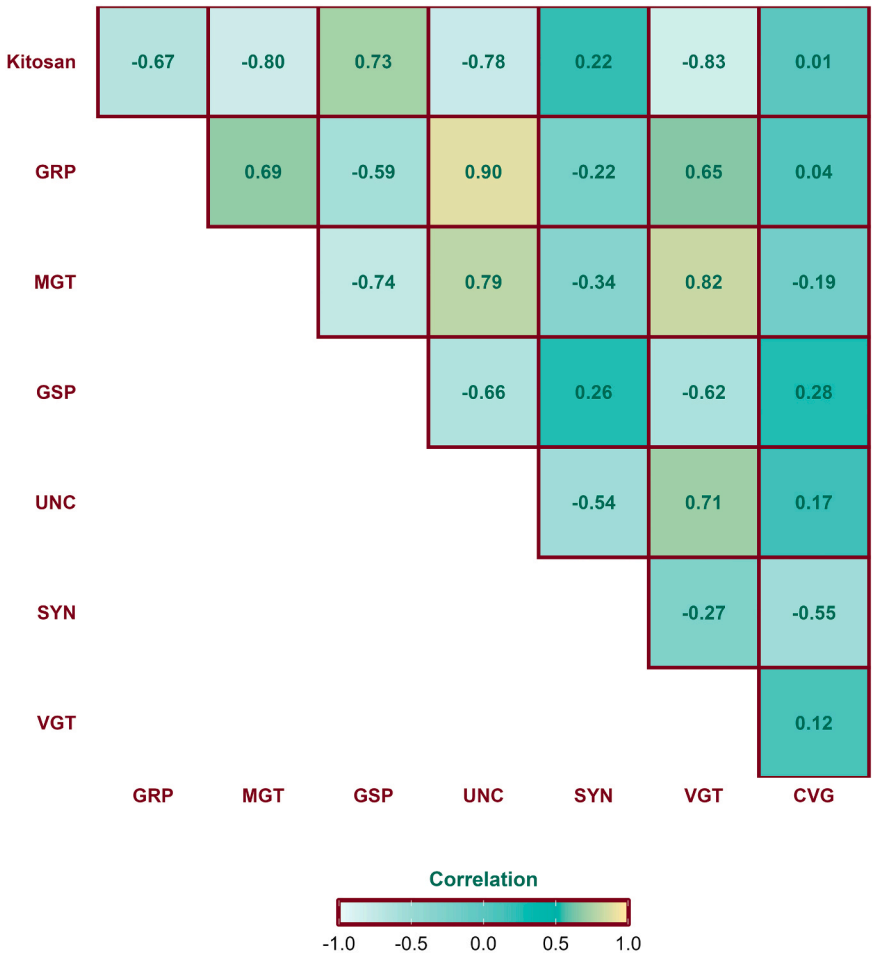

Fig. 2. Heatmap analysis of different CNP concentrations on germination indices of cotton.

Table 2
Impact of CNP concentration on germination indices of cotton.

Chitosan (mg/L)	GRP	MGT	GSP	UNC
0	50.00 ± 0.00	7.20 ± 0.60	14.10 ± 1.28	2.19 ± 0.13
0.25	50.00 ± 10.00	7.93 ± 1.68	13.64 ± 2.42	2.27 ± 0.27
0.5	70.00 ± 10.00	5.31 ± 1.57	21.80 ± 5.09	2.41 ± 0.16
1.0	73.33 ± 8.82	5.67 ± 0.52	17.93 ± 1.66	2.61 ± 0.06
1.5	33.33 ± 3.33	1.75 ± 0.27	59.81 ± 8.82	1.33 ± 0.21
2.0	20.00 ± 0.00	2.83 ± 1.01	51.85 ± 24.29	0.67 ± 0.33
2.5	20.00 ± 0.00	1.00 ± 0.29	122.22 ± 40.06	1.00 ± 0.00
Chitosan (mg/L)	**SYN**	**VGT**	**CVG**	
0	0.03 ± 0.03	44.63 ± 2.93	94.67 ± 11.88	
0.25	0.00 ± 0.00	24.08 ± 3.10	65.17 ± 8.36	
0.5	0.06 ± 0.00	22.46 ± 6.97	93.84 ± 11.29	
1.0	0.03 ± 0.02	23.58 ± 7.80	83.37 ± 12.98	
1.5	0.17 ± 0.10	1.97 ± 0.32	83.50 ± 15.55	
2.0	0.33 ± 0.33	4.17 ± 2.32	47.14 ± 27.22	
2.5	0.00 ± 0.00	1.00 ± 0.50	109.99 ± 31.43	

pronounced, at around 1.5 times more than in the control at both time periods. Similarly, the ARF (Auxin Response Factor) gene was markedly upregulated by the treatment. ARF gene expression almost doubled on the 14th day and tripled at the 21st day of KZ1 concentration, whereas the KZ2 concentration induced a stronger initial response, with expression at day 14 being higher than that at KZ1. At the second point, however, its expression level was like that of KZ1. In contrast, the UGT gene, which is involved in auxin metabolism, displayed a distinct expression pattern. UGT gene expression at 2.0 mg/L CNP was significantly repressed at the 14th day and returned to the same levels as the control at the 21st day, whereas at the KZ2 concentration, expression at the first time point was like the control but was strongly induced, almost tripling by the second time point (Fig. 5).

3.5. Gene expression analysis with Taguchi design

The results of gene expression were also tabulated with Taguchi design (Table 4, Fig. 6). Results of TD revealed the different impact of CNP and days on different auxin genes, considering the rank of S/N ratio and means, which emphasizes robustness by minimizing variability (noise) and maximizing stability under variable conditions. The results of the ARF gene revealed CNP as the most important factor regulating gene expression compared to means, where time was more significant. In contrast, gene expression of AUX and GTU genes was the same, and time was more significant considering the S/N ratio, and vice versa, CNP was more significant according to the means analysis. The graphical results of TD have been presented for ARF (Fig. 5a,b), AUX (Fig. 5c,d), and UGT (Fig. 5e,f). Results confirmed the variable gene expression for means and SN ratio. The different responses of auxin genes can be

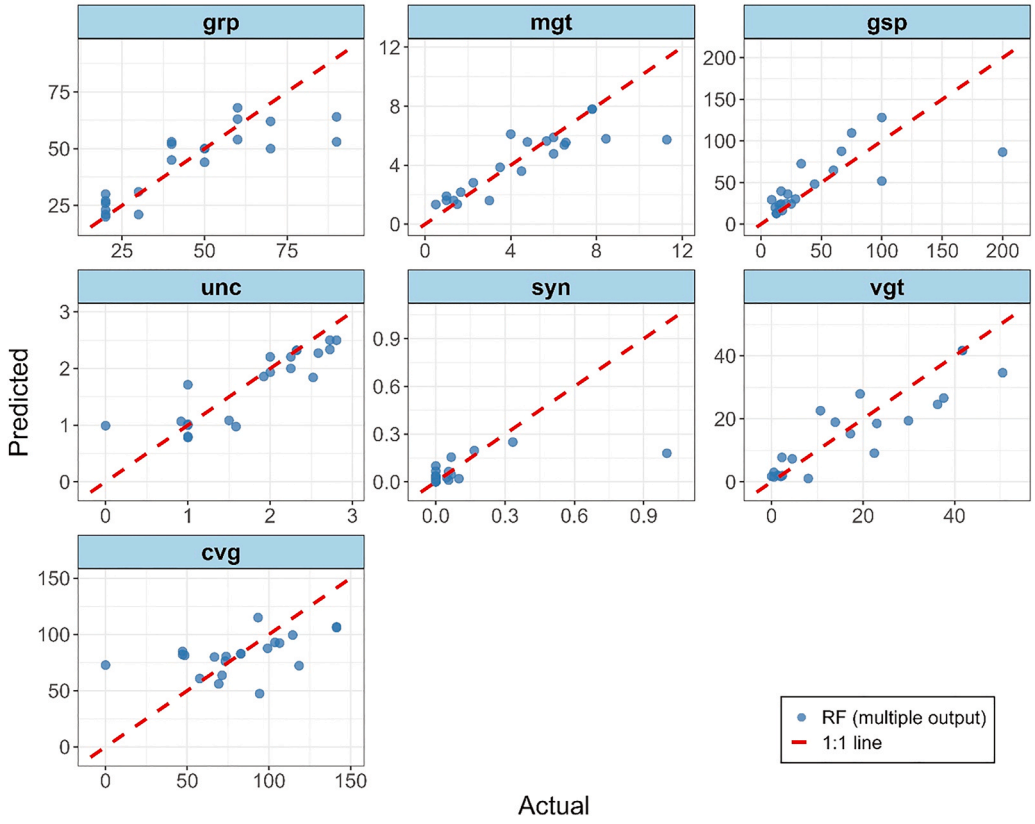

Fig. 3. Random Forest-based Multi-output regression analysis of CNP concentration on germination indices of cotton.

Table 3
Random forest-based multi-output regression analysis of CNP concentration on germination indices of cotton.

Model	R^2	RMSE	MAE
GRP	0.683	12.477	8.619
MGT	0.699	1.578	1.007
GSP	0.512	31.267	18.269
UNC	0.746	0.385	0.279
SYN	0.281	0.185	0.072
VGT	0.785	7.431	5.534
CVG	0.229	28.826	21.991
Mean	0.562		

attributed to the inherent biological differences and their regulatory mechanisms.

4. Discussion

The analysis of germination pattern clearly exhibited the impact of CNP concentration and better germination was recorded at 1.0 mg/L, likely due to enhanced water uptake or enzyme activation during early imbibition (Allam et al., 2024). The germination was relatively slow at lower doses, but still better than the control, which exhibited better metabolic activation by chitosan compared to the control. On the contrary, higher doses slow the germination process, possibly due to higher polymer levels that may have impeded gas exchange or created osmotic stress (Moodi et al., 2024). A similar trend was also observed for the next five days, and 0.25–1.0 mg/L CNPs application was found optimal physiological range for germination. However, further increase of chitosan exerted inhibitory impact on the germination process, likely due to increased viscosity, reduced oxygen diffusion, and excessive polymer coating, resulting in delayed embryo protrusion (Abdel-Aziz, 2019; Allam et al., 2024; Moodi et al., 2024).

Results of germination metrics revealed the impact of CNP concentration, and lower concentration (0.5–1.0 mg/L) resulted in more uniform germination. The results might be due to the influence of low CNP concentration on metabolic activity and the antioxidant defense system (Ling et al., 2022; Bulut et al., 2025). Interestingly, the dual action of CNPs on promoting ot inhibiting germination was observed due to the inverse relationship between MGT and MGR (Veiga-Barbosa and Pérez-García, 2014). Whereas low UNC and VGT scores at higher concentrations indicate more synchronized germination, further confirmed by the low SYN score. Overall results of germination metrics illustrate the role of concentration in stimulating metabolic and physiological processes (Al-Huqail et al., 2018; Wang et al., 2021). The results were further confirmed by heatmap analysis, which illustrated how CNP influences the germination metrics (Sakinah et al., 2021). The GRP and UNC exhibited a strong but positive correlation, confirming the trend of high germination with great variation under stress conditions (Finch-Savage and Bassel, 2016). Similarly, a positive correlation between GRP and MGT illustrated the significance of concentration (Bewley et al., 2012) of the chemical used. Results further exhibited the role of concentration, and a negative correlation between GRP and both MGR and GSP was observed at higher CNP concentration (Wang et al., 2021). The perfect correlation between MGR and GSP illustrates the impact of CNP concentration on biological basis and germination kinetics.

The cotton germination process is highly sensitive and depends on both genetic and environmental factors. Therefore, a multi-output regression model was opted for due to the reason that most of the germination metrics are interdependent and present coordinated physiological responses. Treating them as individuals may overlook antagonistic or synergistic relationships and analyze them collectively (Wang et al., 2024). Whereas the RF model is highly valuable for biological responses that are non-linear, multidimensional, and do not require data distribution (Hoffman et al., 2021; Wang et al., 2024). This modeling

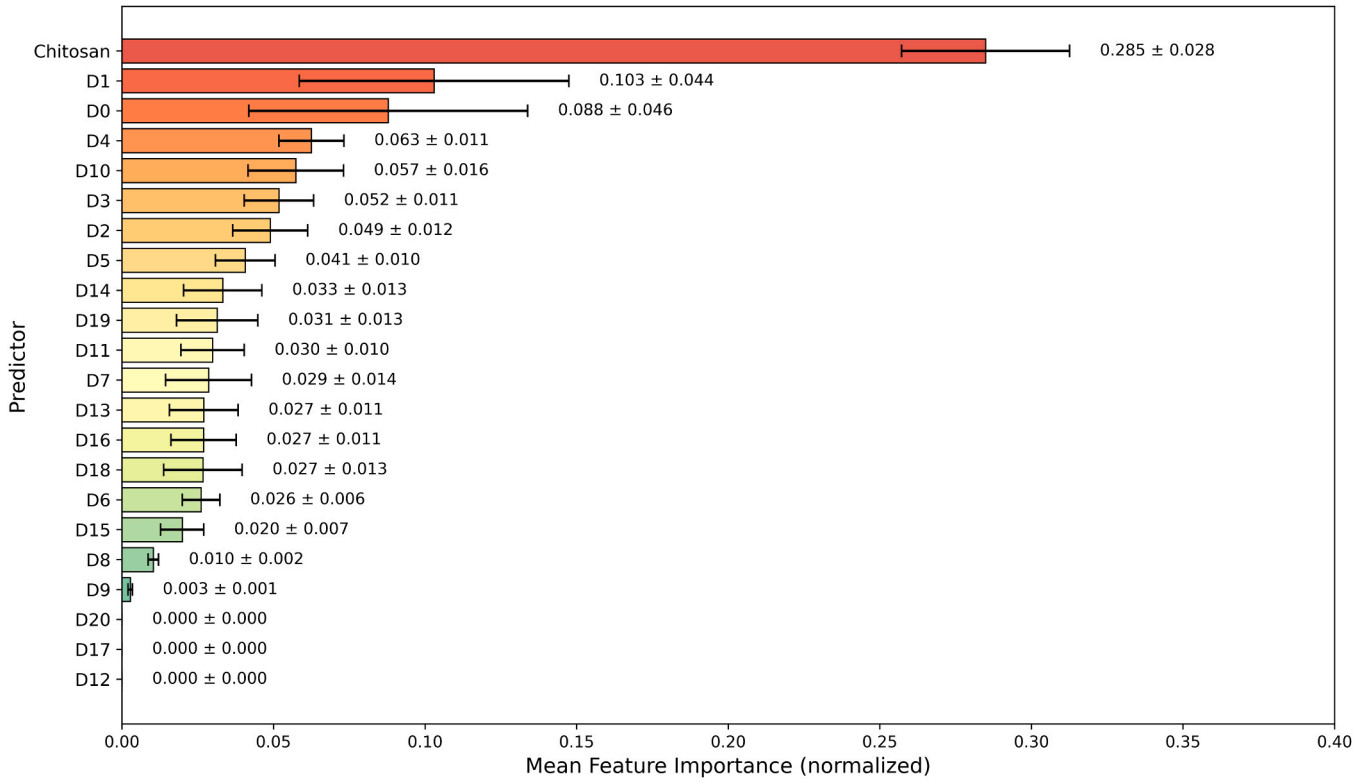

Fig. 4. Feature importance analysis of time series and CNP concntration **Gene Expression Profiling**.

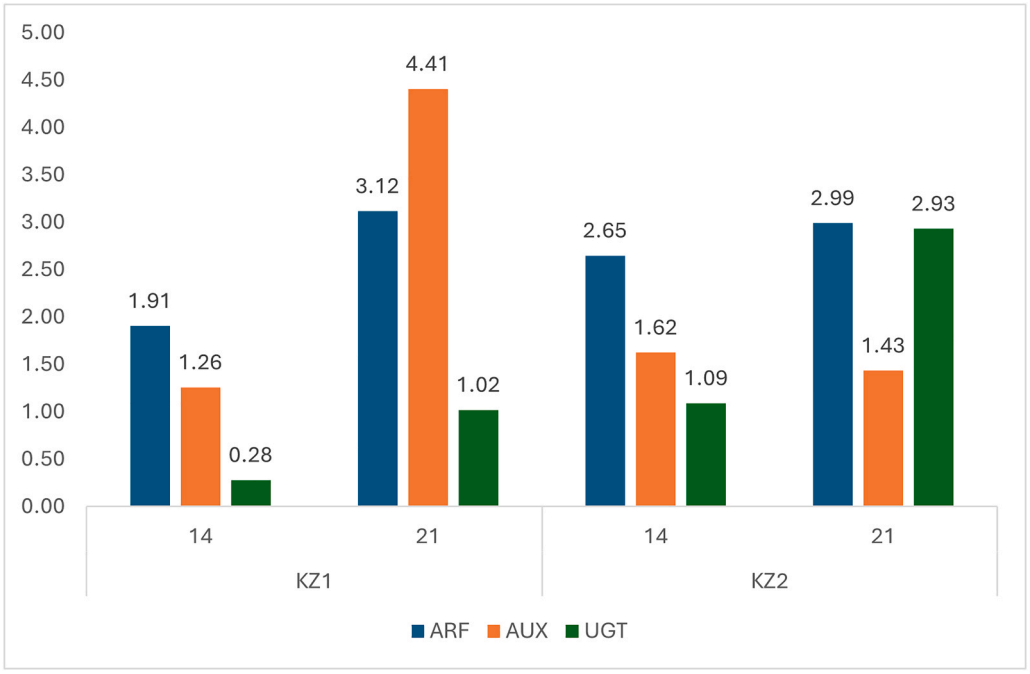

Fig. 5. Relative expression of ARF, AUX, and UGT in cotton seedlings at two chitosan nanoparticle concentrations (KZ1 and KZ2) at two time points (day 14 and day 21).

approach captured shared variance and trade-off between the biological traits. The data on germination metrics computed with an RF-based multi-output regression model revealed reasonable predictions for most of the metrics. The data on germination metrics computed with an RF-based multi-output regression model revealed reasonable prediction for most of the metrics. However, weak prediction for certain metrics like SYN and UNC revealed that these metrics exhibited high variability and sensitivity to germination timing, making them inherently difficult for the model. Results of R^2 scores were further confirmed by the error metrics, indicating that the RF model captured certain metrics successfully. However, the model struggled with metrics directly associated with timing, indicating the need of further predictors or advanced

Table 4
Taguchi design analysis of gene expressions of different auxin genes.

		S/N		Means	
ARF	**Level**	**CNP (mg/L)**	**TIME (d)**	**CNP (mg/L)**	**TIME (d)**
	1	4.195	4.582	3.157	2.685
	2	8.172	7.785	2.979	3.452
	Delta	3.977	3.203	0.178	0.767
	Rank	1	2	2	1
AUX	1	5.227	-1.580	3.079	2.035
	2	0.153	6.960	1.997	3.040
	Delta	5.074	8.540	1.082	1.005
	Rank	2	1	1	2
UGT	1	-7.79	-9.482	0.655	0.868
	2	2.027	3.721	2.273	2.059
	Delta	9.815	13.203	1.618	1.190
	Rank	2	1	1	2

modeling techniques.

The feature importance exhibited a well-defined pattern, with CNP treatment playing a dominant role in driving model predictions. The pattern was consistently stable, with medium phase of D_2 to D_{10} being more dominant compared to later stages. Results confirmed the established phenomenon that early and intermediate germination phased regulates the overall germination process (Ranal and Santana, 2006), indicating their impact on final germination (Aasim et al., 2023). Results further revealed the trait-specific nature of germination response, suggesting the multifaceted nature of germination. However, heterogeneity highlights the adoption of certain model approaches that are capable of capturing the complex biological processes. Results confirmed the significance and suitability of the multi-output regression modeling for capturing the inherent complexity of germination dynamics (Hoffman et al., 2021).

The synthesis of the ARF gene is directly linked to the IAA concentration in plants (Kou et al., 2022). The ARF gene had been significantly overexpressed in the presence of chitosan nanoparticles, with higher expression at later stages, and had a positive correlation with the concentration of chitosan nanoparticles. The distinct upregulation of ARF and AUX at optimal CNP concentrations (1.0 mg/L) likely facilitates the establishment of intracellular auxin maxima required for radical protrusion and seedling establishment. This aligns with findings that chitosan promotes indole-3-acetic acid (IAA) accumulation in roots, enhancing acropetal transport and signaling (Lopez-Moya et al., 2017). The upregulation of ARF at both concentrations, particularly at KZ1 on day 21, suggests enhanced auxin signaling, which aligns with existing studies (Lopez-Moya et al., 2019; Suarez-Fernandez et al., 2020). The AUX gene transcription is known to increase shortly after auxin exposure (Hardtke et al., 2004). In our study, AUX seems to have elevated expression in all treated samples. AUX gene expression had been boosted in the presence of chitosan nanoparticles, with the highest overexpression at the 21st day of KZ1 concentration. The overexpression of AUX was less prominent at the second concentration, with similar patterns at both time points. Increment of chitosan concentration did not affect the expression of AUX in a similar way as with ARF, and at the second time point, expression was like the first time point. The AUX gene expression may be more dependent on IAA concentration than on its presence. The weaker AUX response at KZ2 may indicate a saturation effect, where higher chitosan concentrations do not further enhance auxin influx (Swarup et al., 2008).

UGT converts IAA to indole-3-acetic acid glucose (IAA-glc), lowering the overall concentration of native IAA. Down-regulation of UGT gives more time for IAA to promote growth, whereas overexpression can be a sign of cellular IAA metabolism (Mateo-Bonmatí et al., 2021). Conversely, the dynamic regulation of UGT suggests a dose-dependent homeostatic response. At supra-optimal concentrations (2.0 mg/L), the observed UGT upregulation likely serves to conjugate excess IAA into inactive forms, such as IAA-glucose, to prevent auxin toxicity (Ludwig-Müller, 2011; Mateo-Bonmatí et al., 2021). High doses of chitosan have been reported to repress the quiescent center regulator WOX5 through excessive auxin signaling, leading to root growth arrest (Lopez-Moya et al., 2017). Furthermore, chitosan-induced oxidative stress may trigger specific UGTs, such as UGT74E2, which modulate auxin homeostasis under stress conditions to adapt plant architecture (Tognetti et al., 2010). It has been reported that chitosan triggers plasma membrane depolarization and induces the Tryptophan-dependent auxin biosynthesis pathway, leading to elevated intracellular IAA levels (Lopez-Moya et al., 2017; Suarez-Fernandez et al., 2020). At lower concentrations (KZ1), this induction likely maintains optimal auxin levels for signaling. However, higher concentrations (KZ2) may induce excessive auxin accumulation and secondary messengers like reactive oxygen species (ROS), which can trigger compensatory metabolic pathways (Lopez-Moya et al., 2017). Consequently, the upregulation of *UGT* at higher doses or later time points serves as a homeostatic mechanism to conjugate excess IAA, thereby preventing auxin toxicity and modulating the balance between growth and stress responses (Mateo-Bonmatí et al., 2021). The ANOVA results confirmed thatCNP concentrations were more significant for ARF regulation in cotton. Whereas gene expressions of AUX and UBT in cotton were regulated by time more than by concentration. The comparative result of the S/N ratio with the mean analysis by TD was opposite for all gene expressions. The reason is S/N ratio prioritizes stability and reproducibility rather than prioritizing the magnitude of expression by the mean analysis (Freddi et al., 2019; Kisacik and Aasim, 2025). Results validated that temporal progression plays a regulatory role for these genes, and statistical coordination between ANOVA and TD strengthens the reliability of auxin-gene responses under CNP treatment. The distinct transcriptional profiles of *ARF*, *AUX*, and *UGT* observed in this study provide strong molecular evidence of auxin signaling activation by CNPs. While direct quantification of hormonal levels would provide a downstream metabolic correlate, the significant regulation of these key genes serves as a reliable upstream indicator of the auxin response machinery (Suarez-Fernandez et al., 2020). These results establish a foundational regulatory model for CNP-induced growth, which can be further expanded through multi-omics approaches in future investigations.

5. Conclusion

The results of this study demonstrate the impact of CNP concentration and exposure time on multiple germination metrics and auxin-related gene regulation. The results showed that lower concentrations up to 1.0 mg/L had a positive impact on germination metrics, indicating a biostimulatory effect on early seedling establishment. Whereas higher CNP concentrations resulted in delayed germination kinetics with high variations. The ML analysis using multi-output regression successfully captured the interdependence among germination metrics, and some traits showed high predictive accuracy, indicating their suitability for forecasting multiple traits simultaneously. The molecular analysis of auxin-related genes with qPCR revealed that CNP application enhances the transcriptional activity of key auxin genes (ARF, AUX, and UGT). However, the response of all genes was associated with CNP concentration and exposure time, as confirmed by the TD analysis. Together, these findings provide physiological, molecular, and computational evidence that CNP can serve as a nano-bio stimulator. However, optimum concentration and treatment time are critical, and exceeding these thresholds may induce inhibitory effects. Future studies should focus on optimizing CNP application under diverse environmental and agronomic scenarios, integrating auxin-related gene expression with agronomic traits. Additionally, integrating multi-omics could benefit from broader molecular perspectives to identify upstream regulators, crosstalk pathways, and downstream physiological responses.

CRediT authorship contribution statement

Muhammad Aasim: Writing – original draft, Visualization,

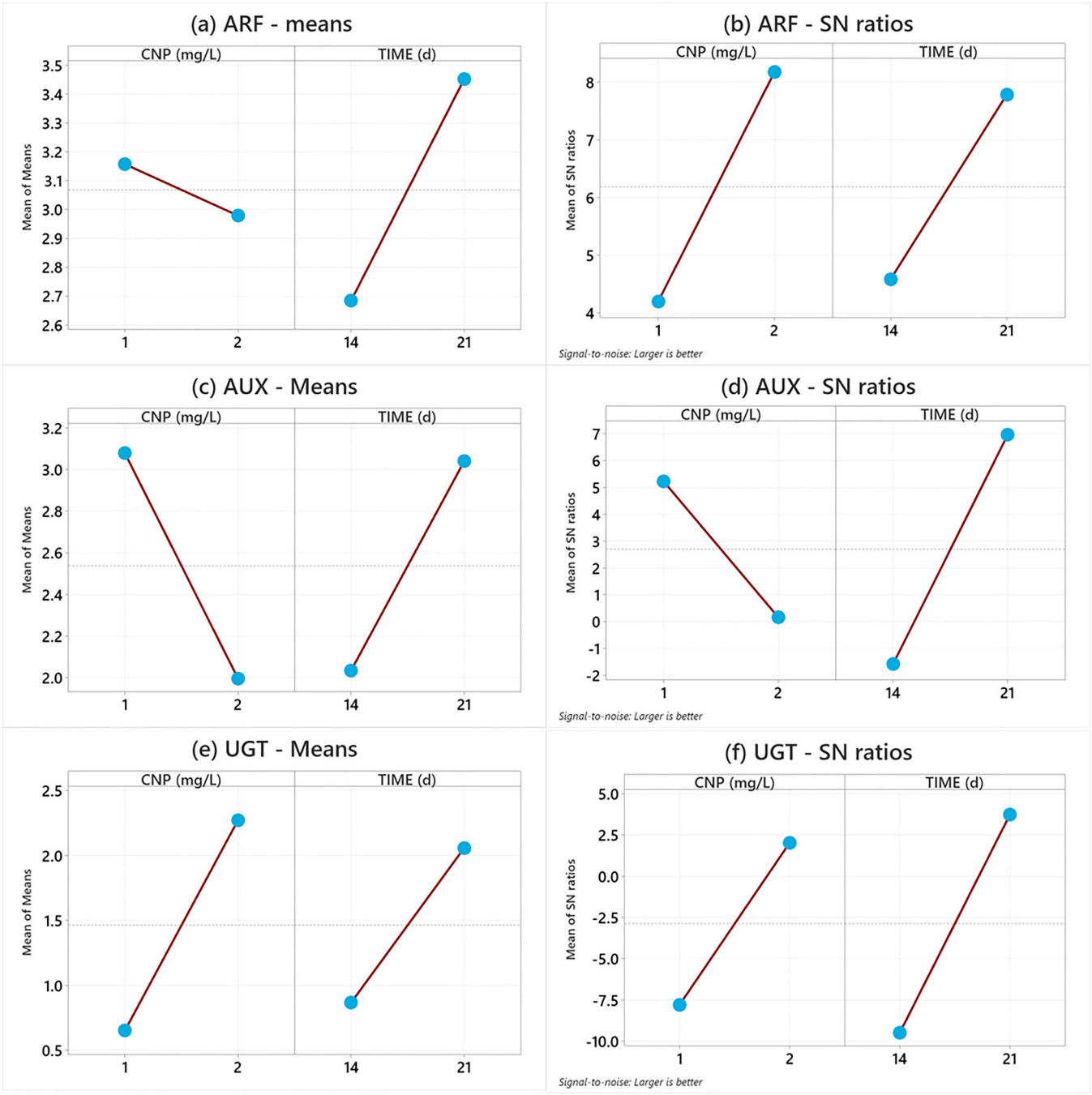

Fig. 6. Comparative analysis of means and SN ratio of gene expressions in response to chitosan treatment and time (a) means for ARF, (b) SN ratio for ARF, (c) means for AUX, (D) SN ratio for AUX, (e) means for UGT, (f) SN ratio for UGT.

Supervision, Methodology, Formal analysis. **Muhammad Tanveer Altaf:** Writing – original draft, Visualization, Formal analysis, Data curation, Funding acquisition. **Zemran Mustafa:** Writing – review & editing, Visualization, Software, Data curation. **Seyid Amjad Ali:** Writing – review & editing, Validation, Software.

Ethical approval

There is no need of ethical approval for the research.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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